Abnormal meiotic behavior in three species of *Crotalaria*

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Abstract – The objective of this work was to compare the meiotic behavior and pollen grain viability of three species of *Crotalaria.* Slides for meiotic analysis were prepared by the air-drying technique. Pollen grain viability was measured by three staining procedures (Alexander's solution, tetrazolium chloride and fluorescein diacetate) and in vitro germination in a sucrose solution. Eight bivalents were observed, confirming previous reports on populations from other regions of Brazil, as well as from other countries. All species showed abnormal meiotic behavior as follows: in *Crotalaria micans*, cytomixis and abnormal chromosome pairing in diakinesis; in *C. spectabilis*, abnormal chromosome pairing in diplotene; in *C. zanzibarica*, shrunk nuclei in leptotene and zygotene. Pollen grains of all three species show low viability, which may be associated with the irregularities of the meiotic behavior.

Index terms: *Crotalaria*, cytogenetics, cytomixis, meiosis, pollen grain viability.

Comportamento meiótico anormal em três espécies de *Crotalaria*

Resumo – O objetivo deste trabalho foi comparar o comportamento meiótico e a viabilidade dos grãos de pólen de três espécies de *Crotalaria*. A análise meiótica foi realizada por meio da técnica de secagem ao ar. A viabilidade dos grãos de pólen foi avaliada por testes de coloração (corante de Alexander, cloreto de tetrazólio e diacetato de fluoresceína) e por teste de germinação em solução de sacarose. Foram observados oito bivalentes, confirmando relatos prévios em populações de outras regiões do Brasil e de outros países. As três espécies apresentaram comportamento meiótico irregular: em *Crotalaria micans*, citomixia e pareamento irregular na diacinese; em *C. spectabilis*, pareamento irregular no diplóteno; e em *C. zanzibarica*, núcleo fortemente condensado nas fases de leptóteno e zigóteno. A viabilidade dos grãos de pólen das três espécies é baixa, o que pode estar associado às irregularidades do comportamento meiótico.

Termos para indexação: *Crotalaria*, citogenética, citomixia, meiose, viabilidade do grão de pólen.

Introduction

Crotalaria L. belongs to the tribe Crotalarieae and is the third largest genus of the subfamily Faboideae (Fabaceae), comprising around 600 herbaceous and shrub species distributed in the tropics and subtropics (Polhill, 1982). In Brazil, there are 31 native and 11 introduced species of *Crotalaria* (Flores et al., 2006). Some are used in agriculture (nitrogen fixation in crop rotating systems and biological control of nematodes); in the paper and fiber industry, in landscaping (Polhill, 1982; Mendonça et al., 1999) and in phytoremediation (Pereira et al., 2002).

Although most of the species of *Crotalaria* are diploid (2n = 2x = 16), some are polyploid, with predominance of tetraploids (2n = 4x = 32), and a few are 2n = 2x = 14 (Mondin et al., 2007). Information on chromosome number, quantitative karyotype parameters, chromosome banding patterns and rRNA gene mapping have been used for phylogenetic and chromosome evolution inferences in *Crotalaria* (Oliveira & Aguiar-Perecin, 1999; Mondin, 2003; Tapia-Pastrana et al., 2005; Almada et al., 2006; Flores et al., 2006; Mondin et al., 2007).

However, little is known on the meiotic behavior of *Crotalaria* species. Verma & Raina (1980) evaluated twenty species and observed chromosome stability based on predominance of normal bivalents, but also related the occurrence of univalents, multivalents, and bridges with or without fragments in some species. These irregularities were considered as evidence for structural changes during the evolution of the genus.

Almada et al. (2006) observed regular meiosis in diploid and polyploid taxa of *Crotalaria*, with ring bivalents as the main configuration in diakinesis. Interestingly, even though some laggard chromosomes

and bridges without fragments were found in all taxa, meiosis was more regular in polyploids than in diploids. The authors considered that polyploid species are probably allopolyploids.

Increasing information on the meiotic behavior of *Crotalaria* species may give important insights on the numerical and structural chromosome changes involved in the evolution of the genus, as shown by Verma & Raina (1980) and Almada et al. (2006).

The objective of this work was to compare the meiotic behavior and pollen grain viability of three species of *Crotalaria*.

Materials and Methods

Flower buds from 25 accessions of three species of *Crotalaria* were collected between November 2007 and March 2008, in two municipalities of Minas Gerais state, Brazil. Vouchers were deposited at the herbarium of Universidade Federal de Lavras (ESAL): *C. spectabilis* Roth, five accessions from Ijaci (21º11'15"S, 44°56'15"W) (ESAL voucher number 22066); *C. zanzibarica* Benth, ten accessions from Ijaci (21º11'15"S, 44°56'15"W) (ESAL voucher number 22067); and *C. micans* Link, ten accessions from Lavras (21º14'43"S, 44°59'59"W) (ESAL voucher number 22070). Duplicates were identified at the herbarium of the Jardim Botânico do Rio de Janeiro. The buds were fixed in Carnoy (3 methanol:1 acetic acid), immediately after collection, and stored at -20°C.

Slides for meiotic analysis were prepared from cell suspensions, according to Viccini et al. (2005), with modifications in the enzymatic digestion of anthers. This step was adjusted for *Crotalaria* using Pectinex Ultra SP-L (Novozymes), at 34°C, in a water bath, for four to six hours. Slides were air-dried, stained with 10% Giemsa solution and evaluated under light microscope (Leica DMLS). All meiotic phases were analyzed and representative figures were digitalized using a digital microcamera (Nikon Digital Sight DS-Fi1). The meiotic index: MI (%) = 100(number of normal tetrads/total meiocytes) was calculated from 5,000 meiocytes per species.

Pollen grain viability was evaluated using three staining methods: Alexander's solution, for 24 hours with fixed and fresh material, at 4ºC; 2,3,5-triphenyltetrazolium chloride in 5 (TTC 5%) and in 50% (TTC 50%) sucrose solution, for 2 hours with fresh material, at room temperature in the dark; and fluorescein diacetate (FDA) 6.25 µg mL^{-1} in 25% sucrose solution, for 30 min with fresh material, at room temperature in the dark. Slides were prepared by squashing the anthers (fresh or fixed) in a drop of staining solution. In each method, percentage of viable pollen grains was obtained from 1,000 mature microsporocytes observed in five slides per species. Slides for the Alexander and TTC methods were evaluated under a light microscope (Leica DMLS), while those for FDA were evaluated under epifluorescence using an Olympus BX60 microscope (460–490 nm excitation and 515–550 nm emission filter). Fresh pollen grains were cultured in liquid media containing sucrose at 15, 20 and 50% for *C. spectabilis*, *C. micans* and *C. zanzibarica*, respectively, for 24 hours, in a humid chamber, at 28°C. All pollen grains from five slides per species were analyzed under a light microscope (Leica DMLS) to estimate germination percentage.

Results and Discussion

Synchrony of flower bud size and meiotic phases was observed. The best interval of flower bud size to obtain meiocytes was 6.5 to 7.0 mm for *C. zanzibarica*; 4.5 to 5.0 mm for *C. micans* and 5.5 to 6.0 mm for *C. spectabilis*. All three species showed n = 8 chromosomes (Figure 1), confirming previous reports

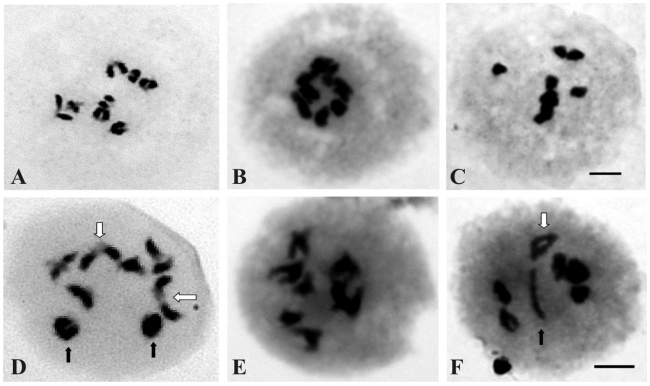

Figure 1. Chromosome number (n = 8) of *Crotalaria* species. (A) Diakinesis in *C. zanzibarica*; (B) metaphase I in *C. micans*; (C) metaphase I in *C. spectabilis*; (D) rod (white arrow) and ring (black arrow) bivalents in *C. zanzibarica*; (E) bivalents in *C. micans*; (F) rod (black arrow) and ring (white arrow) bivalents in *C. spectabilis*. Bar: 5 µm.

for *C. zanzibarica* (Atchinson, 1950; Yeh et al., 1986), *C. micans* (Almada et al., 2006; Flores et al., 2006) and *C. spectabilis* (Mondin, 2003; Almada et al., 2006).

Ring and rod bivalents were detected in 0.33% diakineses for *C. zanzibarica* (Figure 1 D) and in 0.73% diakineses for *C. spectabilis* (Figure 1 F). In the latter species, ring pairing was more frequent as also observed by Almada et al. (2006). In *C. micans*, neither rod nor ring bivalents were observed (Figure 1 E), while Almada et al. (2006) described their occurrence.

All three species had irregular meiotic behavior with different kinds of abnormalities. In *C. zanzibarica*, approximately 14% of the meiocytes were irregular. Of these, 89.19% were in prophase I, presenting strongly condensed nuclei, different from the normal leptotene and zygotene pattern (Table 1 and Figure 2 A). Sticky chromosomes (Figure 2 B), laggard or lost chromosomes in metaphase I and prophase II (Figure 2 C and D), irregular meiotic spindle (Figure 2 E), and triads with micronuclei (Figure 2 F) were also observed.

In *C. micans*, ca. 8% of the meiocytes were irregular, presenting: sticky chromosomes (Figure 3 A); laggard or lost chromosomes in metaphase I (Figure 3 B); irregular meiotic spindle in metaphase II and asynchronic telophase II (Figure 3 C and D). Laggard or lost chromosomes and bridges in anaphases I and II were reported by Almada et al. (2006).

This species also showed migration of genetic material between cells, evidencing the occurrence of cytomixis (Figure 3 E to I), which corresponded to 16% of the total irregularities. Cytomixis was observed only in prophase I, corresponding to 11.08% of the irregularities in leptotene/zygotene and 4.92% of those in pachytene. Ocurrence of cytomixis during prophase I, mainly in pachytene, has been reported in species of Fabaceae (Wang et al., 2002; Belluci et al., 2003; Haroun et al., 2004; Sidorchuk et al., 2004).

Partial or complete chromatin migration involving cytomitic cells was observed (Figure 3 G), with some acting as donor and others as receptor meiocytes (Figure 3 H and I). Some authors suggested that chromatin and chromosome migration is not random but directional. For example, Falistocco et al. (1995) observed that, in *Dactylis*, cytomixis always occurred from a donor to a receptor cell and that, in several cases, complete or almost total genetic material migration occurred not only between two cells, but also amongst several cells at the same time.

Table 1. Percentage of irregularities observed per meiotic phase of *Crotalaria* species.

Phase	Abnormal meiocytes (%)		
	C. zanzibarica	*C. micans*	*C. spectabilis*
Leptotene Zygotene	89.19	15.13	0.00
Pachytene	0.00	7.65	1.60
Diplotene	0.29	10.10	64.28
Diakinesis	0.73	45.56	8.30
Metaphase I	1.63	4.04	2.72
Anaphase I	2.96	1.01	1.33
Telophase I	0.29	3.03	3.28
Prophase II	1.22	0.00	0.00
Metaphase II	0.81	3.03	4.11
Anaphase II	0.60	1.01	0.77
Telophase II	0.50	2.02	2.72
Tetrad	0.00	6.06	0.77
Triad	0.50	2.02	1.60
Undefined	1.63	-	8.85

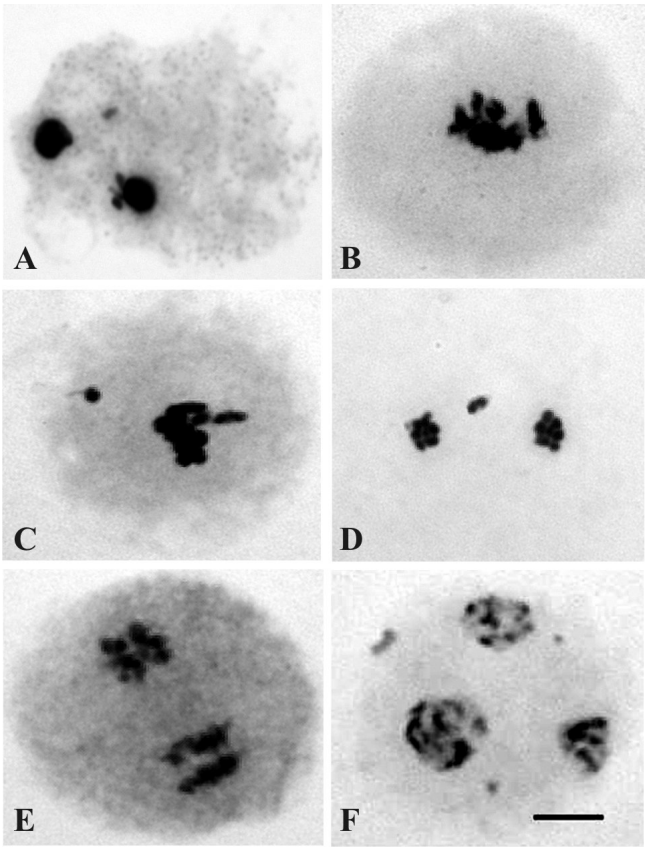

Figure 2. Meiotic abnormalities in *Crotalaria zanzibarica* (n = 8). (A) Condensed nuclei; (B) sticky chromosomes; (C) metaphase I; and (D) prophase II with laggard or lost chromosomes; (E) irregular meiotic spindle; (F) triad with micronucleus. Bar: 5 μm.

Cytomixis in meiosis can have important implications in evolution, as it can lead to the formation of unbalanced gametes, including non-reduced ones. If these gametes are viable, they will originate aneuploid or polyploid plants. This was suggested by Belluci et al. (2003) working with *Medicago sativa*. High pollen grain viability (87.1 to 95.1%) of *Dactylis* plants showing cytomixis was reported by Falistocco et al. (1995), evidencing that cytomixis can be potentially important in the production of non-reduced pollen grains (2n).

In *C. spectabilis*, approximately 12% of the meiocytes were irregular. There was a high percentage of irregular diplotene (64.28%) showing multivalents (Figure 4 A). There were also: sticky chromosomes (Figure 4 B); early chromosome segregation in metaphase I (Figure 4 C); laggard chromosomes in prophase II (Figure 4 D); irregular meiotic spindle in metaphase II (Figure 4 E) and telophase II, as well as chromosome asynchronism and micronuclei (Figure 4 F). In populations of *C. spectabilis* from Argentina, Almada et al. (2006) found laggard or lost chromosomes and bridges, but all tetrads were normal.

Each species showed a predominant type of irregularity. Most of the abnormal meiocytes in *C. zanzibarica* had strongly condensed nuclei in prophase I. *Crotalaria spectabilis* and *C. micans* presented multivalents and, in the latter, cytomixis was also observed (Table 1). However, meiotic index values were high: *C. zanzibarica*, 92.19%; *C. micans*, 91.32%; and *C. spectabilis*, 88.18%.

Pollen grain germination in the analyzed species was low. Similar results were obtained in the pollen viability tests using fluorescein diacetate (FDA) and

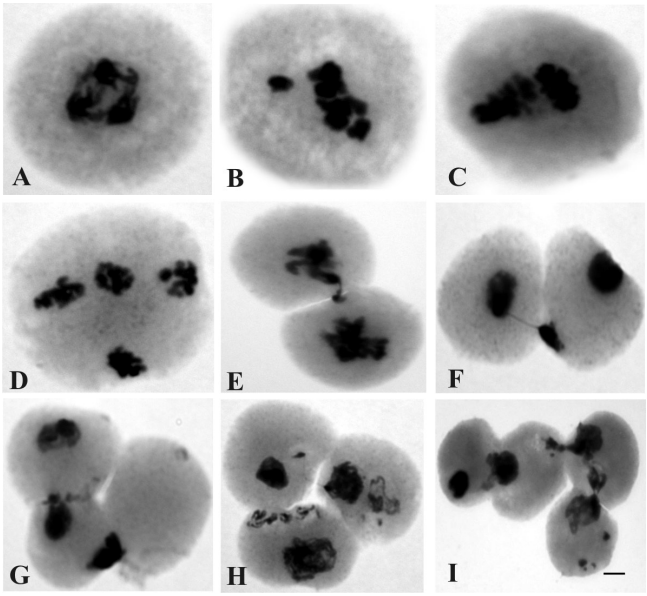

Figure 3. Meiotic abnormalities in *Crotalaria micans* (n = 8). (A) Sticky chromosomes; (B) metaphase I with laggard chromosomes; (C) metaphase II and (D) asynchronic telophase II with irregular spindles; (E) cytomixis between meiocytes at the beginning of pachytene; (F) meiocytes in zygotene with simple chromatin bridge; (G) total chromatin migration from one meiocyte to another in prophase I; (H–I) chromatin migration among three or more cells in prophase I, with micronuclei. Bar: 5 µm.

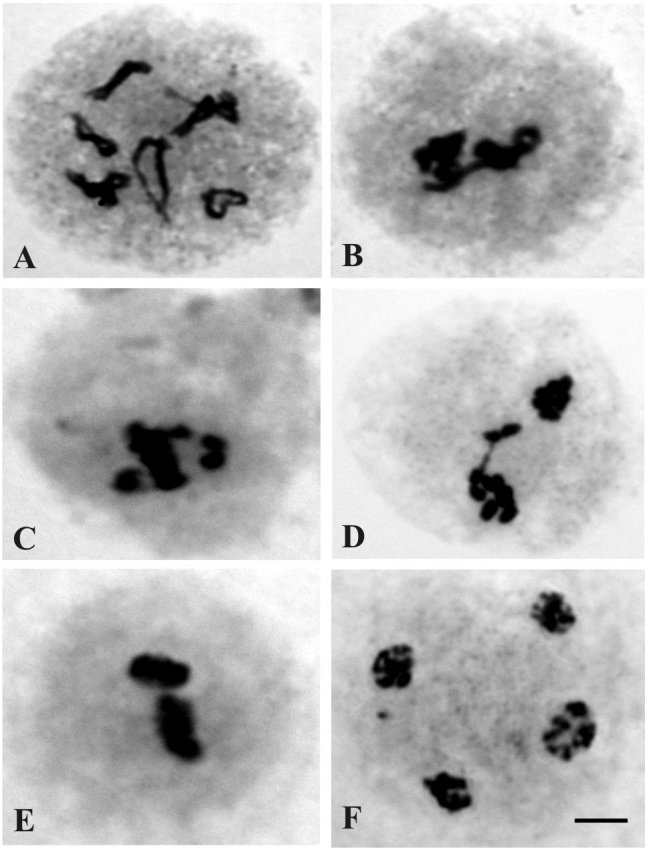

Figure 4. Meiotic abnormalities in *Crotalaria spectabilis* (n = 8). (A) Diplotene with multivalents; (B) sticky chromosomes; (C) early chromosome segregation in metaphase I; (D) laggard chromosomes in prophase II; (E) irregular spindle in metaphase II; and (F) asynchronic telophase II, including micronucleus. Bar: 5 µm.

2,3,5-triphenyltetrazolium chloride (TTC) – 50%, indicating low pollen viability (Table 2 and Figure 5). Staining tests using Alexander's solution, with both fixed and in natura materials, showed high percentage of viable pollen grains (Figure 5), followed by the test using TTC – 5% (Table 2). This could be explained by the incapability of Alexander's solution and TTC – 5% to detect the low pollen viability shown in the germination test. These results show that FDA and TTC – 50 % are reliable tests for pollen grain viability studies in *Crotalaria*.

Low pollen grain viability could be either associated to the observed meiotic irregularities, since they have potential for chromosome number alteration, or to post-meiotic events. Still, considering that these species produce large amounts of seeds, both in natural populations and under cultivation, such low viability must be compensated by the large number of pollen grains.

Table 2. Pollen grain viability percentage determined by staining tests and in vitro germination percentage (G) of *Crotalaria* species.

Species	Pollen grain viability					G
	A1[(1)]	A2[(1)]	TTC 5%[(2)]	TTC 50 %[(3)]	FDA[(4)]	
C. zanzibarica	98.6	96.4	56.8	7.0	7.2	6.9
C. micans	98.0	98.7	57.2	13.7	14.8	14.5
C. spectabilis	99.3	98.7	62.0	11.4	28.4	17.4

[(1)]Alexander's solution in fixed (A1) and fresh (A2) pollen grains. [(2)]5% 2,3,5-triphenyltetrazolium chloride in 5% sucrose solution. [(3)]5% 2,3,5-triphenyltetrazolium chloride in 50% sucrose solution. [(4)]Fluorescein diacetate 6.25 µg mL^{-1} in 25% sucrose solution.

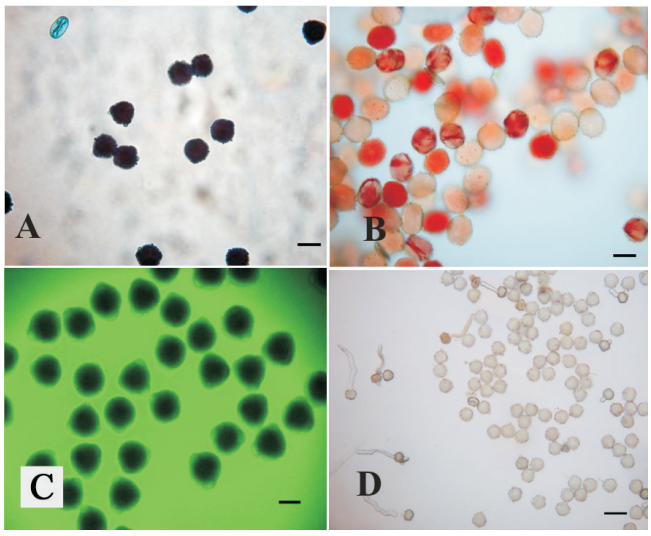

Figure 5. Viability of *Crotalaria* sp. pollen grains revealed by staining and germination tests. (A) Fresh pollen grains of *C. spectabilis* stained with Alexander's solution; intact microsporocytes with deep purple cytoplasm are viable, while the fragmented ones with weak green cytoplasm are inviable. Bar: 20 µm. (B) Fresh pollen grains of *C. micans* in 5% 2,3,5-triphenyltetrazolium chloride in 50% sucrose solution; deep red microsporocytes are viable, and colorless ones, inviable. Bar: 20 µm. (C) Fresh pollen grains of *C. spectabilis* stained with fluorescein diacetate (FDA); nonfluorescent microsporocytes are not viable. Bar: 20 µm. (D) *C. spectabilis* pollen grains in germination inducing liquid medium. Bar: 50 µm.

Conclusions

1. *Crotalaria zanzibarica*, *C. micans* and *C. spectabilis* have irregular meiotic behavior, with predominance of shrunk nuclei in leptotene and zygotene in *C. zanzibarica*, cytomixis and abnormal chromosome pairing in *C. micans* and abnormal chromosome pairing in *C. spectabilis*.
2. Abnormal meiotic behavior does not affect the tetrad production.
3. Viability of pollen grains of *C. zanzibarica*, *C. micans* and *C. spectabilis* is low.

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